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Regulatory Status of Transgenic American Chestnut Tree Under FIFRA

Developing an American Chestnut that Tolerates the Chestnut Blight Fungus

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Abstract *The American Chestnut Research and Restoration Project at the College of Environmental Science and Forestry (ESF) has transformed an American chestnut* (Castanea dentata) *with the addition of an oxalate oxidase gene found in wheat and other common food crops. This transgenic (TG) tree can coexist with the fungus that causes chestnut blight* - Cryphonectria parasitica. *The fungus is an invasive species that all but wiped out the historic American chestnut population during the first half of the last century. ESF's intent, working with The American Chestnut Foundation and other partners, is to restore the American chestnut to its former range with the transgenic tree and regional outcrosses. The TG American chestnut can survive in the presence of the now ubiquitous and lethal* C. parasitica *fungus because the TG tree expresses an enzyme that neutralizes the oxalic acid released by the fungus that otherwise would damage the tree and eventually kill it. The enzyme is not intended to have and has no direct effect on the fungus itself, which continues to live and reproduce on the TG chestnut. The intent of the transformation is only to minimize physical damage to the tree from the acid. The question has been raised whether the TG chestnut is subject to comprehensive, life cycle regulation as a "pesticide" under the Federal Insecticide, Fungicide and Rodenticide Act (FIFRA). In our view, it is not a "pesticide" because there is no intent to mitigate a pest. The added gene is not intended to, and does not destroy, repel or mitigate the fungus itself (the pest); it is only intended to minimize physical damage to the tree from the inanimate acid released by the fungus. Nor is the intent to control the growth of the fungus by destroying its habitat. The technology is effective regardless of the continuing presence of the fungus, and does not depend on affecting the growth of the fungus.*

Introduction.

The American chestnut (*Castanea dentata*) was historically a dominant forest overstory species that performed myriad ecologically important functions throughout the eastern woodlands of North America. Because it could grow rapidly and attain huge sizes, the tree was often the outstanding visual feature in both urban and rural landscapes. Starting around 1900, the American chestnut fell victim to an invasive fungus that was inadvertently introduced by human activity. The fungus reduced the American chestnut from its position as the dominant tree species in the eastern forest to little more than an early-succession-stage shrub. There has been essentially no chestnut lumber sold in the United States for several decades and the bulk of the annual 20-million-pound chestnut nut crop now comes from introduced chestnut species or imported nuts. The American chestnut has not gone extinct. The species

has survived by sending up stump sprouts that grow vigorously in logged or otherwise disturbed sites, but inevitably succumb to the fungus and die back to the ground. There is essentially no hope of avoiding the disappearance of the American chestnut in the wild without significant human intervention. The technology described below is a key component of efforts to rescue this species, one of the most complex and prolonged single-species conservation tasks ever attempted (Steiner et al. 2017).

Cryphonectria parasitica (*C. parasitica*) is the invasive fungus that kills the American chestnut. The fungus initially infects a tree by entering and colonizing wounds in the bark where it absorbs nutrients from dead or dying tissue. The fungus also releases oxalic acid (forming oxalate salts), which increases the damage to the tree, killing bark, cambium, and wood tissues of the tree stems and releasing additional plant cell contents, which the fungus can use in its own metabolism. This damage is evidenced by areas of dead cells (necrotic canker or blight). Where the innermost bark layer (phloem) is damaged or killed, it can no longer transport sugars from the leaves to the tree roots. If this damage encircles and girdles the tree, the portion of the tree above the infection soon dies.

Importantly, while the released oxalic acid increases the damage to plant cells, the fungus is not dependent on this mechanism to thrive. Even without any oxalic acid, *C. parasitica* invades wounds, colonizes tissue and replicates on American chestnuts (Chen et al., 2010). Indeed, in its natural range, *C. parasitica* colonizes the more highly resistant Chinese chestnut (*Castanea mollissima*) upon which it thrives as a saprophyte and weak parasite (Clapper, 1952), and produces only superficial cankers that do not kill the tree (Hebard et al., 1984). In North America, the fungus similarly survives on several endemic species of oaks (*Quercus spp.*), hickory, sumac, chinquapin, and red maple, which are less susceptible to oxalic acid damage than the American chestnut (Nash & Stambaugh, 1987, Stipes et al. 1978, Davis et al., 1997, Baird 1991). The fungus also survives even more abundantly on dead American chestnut stems as a saprophyte than on a live American chestnut (Prospero, et al. 2006). Perpetual colonization of these other forest species by *C. parasitica* is a principal reason that the fungus (and associated chestnut blight) remains prevalent in North America, even as the American chestnut itself has nearly disappeared as an overstory, canopy tree species from eastern woodland and is considered "functionally" extinct.

Modified Chestnut Tolerates Presence of *C. parasitica* Fungus

To help restore the American chestnut, the ESF research team has produced American chestnut trees modified to tolerate and coexist with the ubiquitous *C. parasitica* fungus. We used *Agrobacterium*-mediated transformation to add wheat gene gf-2.8 to the American chestnut genome.[1] This gene encodes for the enzyme oxalate oxidase (EC 1.2.3.4), or OxO, which catalyzes the degradation of the oxalic acid produced by the growing fungus. OxO detoxifies the acid by degrading (or oxidizing) it into carbon dioxide and hydrogen peroxide, effectively neutralizing some of the acid and minimizing the physical damage to the tree that might otherwise occur due to the presence of this acid. This allows the fungus and the tree to coexist, just as the fungus coexists with the naturally blight-tolerant Chinese chestnut trees from its native range. The enzyme does this without mitigating the fungus.

The OxO enzyme is not intended to and does not kill or otherwise directly affect the fungus at all. The intent is only to neutralize the inanimate acid after the fungus has released it so that the acid does not damage tissues that eventually kill the tree. Nor is the OxO enzyme intended to mitigate the fungus by destroying the habitat where the fungus might otherwise grow. The technology achieves its purpose — preventing the death of the tree that results from the acid damage to the tree's phloem (or conductive tissues) — regardless of the continuing presence of the fungus. It does not need to resist ongoing colonization or reproduction by the fungus to be effective. In fact, the transgenic (TG) tree remains a suitable food source for the fungus. The *C. parasitica* fungus thrives on the TG tree just as it does on damage-resistant Asian chestnuts and other tree species.

This relationship is demonstrated by an earlier version of the TG American chestnut ('Darling 4') that has been growing in the open environment in the New York Botanical Gardens in Bronx, NY (under an

[1] This transformation vector is classified as a "plant pest" (7 CFR §340.2) making the TG chestnut a "regulated article" (7 CFR §340.1) subject to APHIS control of its movement and environmental release, unless, after risk assessment, APHIS determines that the TG tree is not a plant pest and therefore should not be regulated. 7 CFR §340.6.

APHIS permit) for over five years. **Figure 1** is a close-up picture of the trunk of this 'Darling 4' tree. As shown, the the fungus is growing on the tree, forming cankers that damage the stem tissues, but, as shown in the **Figure 1 inset**, the tree survives.

This is an example of coexistence. The newer versions of the TG American chestnut ('Darling 54' and 'Darling 58') express the OxO enzyme at higher levels and more broadly, and are more effective in neutralizing the oxalic acid and minimizing acid damage to the tree. However, the fungus still colonizes and reproduces on on TG trees principally in saprophytic form instead of exerting parasitic effect. **Figure 2** shows small stem assays of a naturally blight tolerant Chinese chestnut ('Qing') and TG American chestnut ('Darling 54'), each infected with *C. parasitica*. The fungus can be isolated from the wounded and dead bark of the tree, also shown in **Figure 2**. These trees survive and coexist with the fungus.

We have often referred to the function of the OxO enzyme as enhancing "blight resistance" in the TG tree. While this is true, the phrase is ambiguous and may be misunderstood. The term "blight" correctly refers to the physical damage caused to a plant by a pest, but it is sometimes applied to the pest itself. In many contexts, the distinction is not important. Similarly, the general term "resistance" is broad and can be used to describe an array of methods by which a plant can "resist" pest damage. These range from changes to the plant that, at one end of the spectrum, kill the pest or prevent it from reproducing, to, as in this case at the other end of the spectrum, changes to the tree that allow it simply

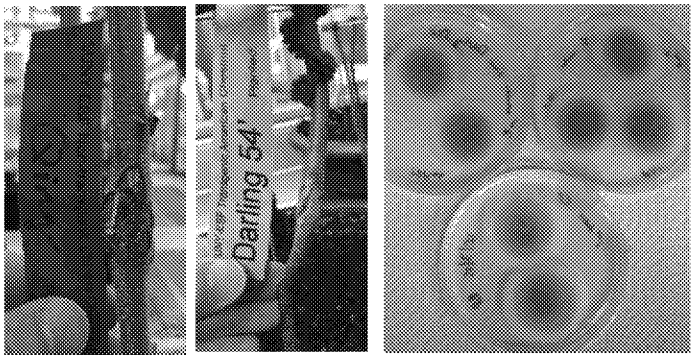

Figure 2. *Small stem assays of trees infected with* C. parasitica. *Left, blight tolerant Chinese chestnut ('Qing'). Center, oxalate oxidase-expressing American chestnut ('Darling 54'). Right,* C. parasitica *isolated from the margins of the cankers. These trees survived the infection and coexist with the fungus.*

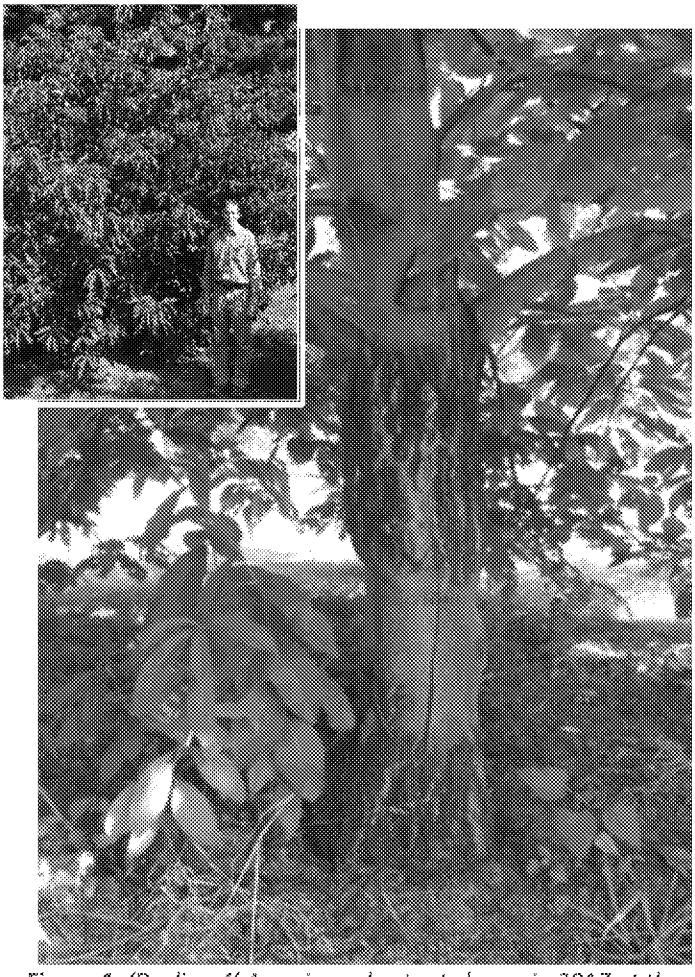

Figure 1 *'Darling 4' American chestnut shown in 2017 at the New York Botanical Gardens, Bronx, NY. It exhibits several large cankers (two shown) caused by* C. parasitica. *It was planted in 2012.*

to tolerate the continuing presence of the pest without necessarily affecting the pest.

In the case of the TG American chestnut, the "resistance" is based on tolerance, where the pest is not harmed, but instead the host is changed so that it can tolerate the pest's continuing presence as it would be in its native environment on Chinese chestnut trees. Therefore, a more precise definition for our TG tree would be a "blight tolerant" American chestnut tree.

The continuing presence of *C. parasitica* on the TG tree has the added benefit of reducing selective pressure for the fungus to overcome the action of the OxO enzyme. Here, the TG tree's "tolerance" mode of action, in which the fungus continues to colonize and reproduce and the fungus and tree coexist, benefits both organisms.

Consistent with this mode of action, if the TG tree were branded for distribution, the tree tag might read as shown in **Figure 3**.

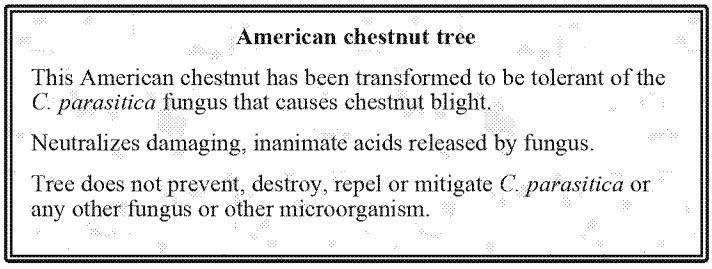

Figure 3: *Mock-up of possible tag to accompany distribution of TG tree.*

Regulatory Status of TG American Chestnut Under FIFRA.

Is the TG American chestnut a "pesticide" subject to registration and other regulation under the Federal Insecticide, Fungicide and Rodenticide Act (FIFRA)? In practical terms, this is a very consequential question. The initial registration of a pesticide substance and product is an uncertain and very costly, multi-year process, and a major undertaking. Once a pesticide is registered, additional state registrations are normally required in each state where the product will be distributed. The product is subject to ongoing labeling and reporting requirements, and, to maintain the registration, it is subject to periodic review by EPA, including (typically) follow-up data development (testing) obligations for registrants. This oversight regime is not inappropriate for "economic poisons" that rely directly or indirectly on toxicants to mitigate pests. But it is unwarranted for products that do not involve toxicants or pesticidal claims, and it is inapplicable to products intended to mitigate inanimate pest damage but not intended to mitigate animate pests.

Legal Test for FIFRA Pesticide Regulation. FIFRA only regulates "Pesticides," which are defined as a "substance" "*intended* for preventing, destroying, repelling, or mitigating any *pest*[.]" FIFRA §2(u); 40 CFR §152.3 (emphasis added). Only a form of life (*e.g.*, a fungus) can be a pest. FIFRA §2(u). Pesticide substances can act directly on the pest (*e.g.*, as a toxicant). A substance intended to indirectly destroy or mitigate a pest may also be a pesticide, such as where there are claims that the product affects the habitat or food source of a pest with the intent to mitigate the pest itself. But there is no FIFRA jurisdiction where the intent is only to neutralize or remove *inanimate* pest residues, without claims or intent to mitigate the pest itself, even if there is, in fact, some predictable but incidental mitigation of the pest (*e.g.* surface cleaners intended to remove fungus stains or odors, with no claim to remove or control the fungus itself). 40 CFR §152.10 ("Products that are not pesticides because they are not intended for a pesticidal purpose").

Accordingly, in this case, in order for the OxO gene and enzyme to be regulated as pesticides, a human would have to intend that these substances destroy, prevent, repel, or mitigate the animate *C. parasitica* fungus itself, either directly, or by destroying its habitat or food source.

The OxO Enzyme Is not a Pesticide. The OxO enzyme, as we are using it, does not fit the definition of a pesticide. The intent is only to neutralize the inanimate oxalic acid released by the fungus to minimize (lessen) the *damage* to the tree, with no intended or claimed pesticidal effect (destroy, repel, mitigate) on the fungus itself. In fact, *C. parasitica* continues to colonize and reproduce on the TG tree in the presence of OxO, much as it would in its native environment on Chinese chestnut trees. *See Figures 1-3 and accompanying text.*

The relatively smaller acid damage (cankers) seen in TG trees does imply that there will be relatively less readily available (dead) plant cell contents for the fungus to use in its own metabolism and arguably less fungus growth than would be expected in a wild-type chestnut tree. But this fact does not change the conclusion that the OxO enzyme is not a pesticide. As an initial matter, the fungus is not dependent on this mechanism to thrive. Even without any oxalic acid, *C. parasitica* infects wounds, colonizes tissue and replicates on American chestnuts. More importantly, there is no intent to mitigate the growth of the fungus by depriving it of nutrients. The success of the OxO technology (minimizing damage) does not depend on reducing the amount of fungus on the tree; that is not the intended or actual mode of action. Because OxO is expressed throughout the tree, the TG tree will continue to survive regardless of the amount of fungus. From a product intent perspective, we are indifferent to the amount of fungus present on the TG tree. Indeed, if anything, the preference is for the fungus to remain alive on the tree to minimize any evolutionary pressure.

This mode of action and intent sets the OxO technology apart from pesticides whose intended mode of action is to "mitigate" pests by destroying their habitat or food source so they cannot grow. The intent of the OxO technology is more akin to that of a fungus *stain* cleaning or deodorizing product with only cleaning and deodorizing claims. Their use may in fact kill pests or make the pest's potential habitat inhospitable. While those effects are predictable, they are also only incidental to the non-pesticidal cleaning intent. The intent of the OxO technology is also comparable to the intent of pruning paints that prevent pest damage by denying pests access to nutrient sources in plant wounds with a physical barrier. As in the OxO case, pests excluded by the paint are not affected directly, but they may not spread and thrive to the same extent on the paint-protected plant as they theoretically would on an unprotected plant. Nevertheless, even though predictable, the paints are not pesticides due to any such indirect "mitigation" because this effect is unintended and only incidental to the intended mode of action (damage prevention).

To avoid doubt, EPA regulations include a three-part test to determine the "intent" of a product. A substance is considered to be intended for a pesticidal purpose if (40 CFR §152.15):

(a) The distributor claims or implies that it can be used as a pesticide; or

(b) The substance contains an ingredient capable of mitigating a pest and has no significant commercially valuable use other than for pesticidal purposes; or

(c) The distributor knows that the substance will be used, or is intended to be used, for a pesticidal purpose.

Here, the OxO enzyme and gene do not trigger any of the applicable tests: (a) we do not claim (and indeed expressly disclaim) that the product can be used to mitigate *C. parasitica*; (b) OxO cannot directly mitigate *C. parasitica* and any arguable indirect pest mitigation from OxO (as opposed to acid mitigation) is insubstantial and only incidental to its intended non-pesticidal use to neutralize acid and avoid damage; and (c) there is only one possible use of OxO and it is ineffective as a pesticide. *C. parasitica* continues to colonize and reproduce on the TG tree in the presence of OxO, much as it would in its native environment on Chinese chestnut trees.

Even if the OxO enzyme directly affected C. *parasitica*, it would be appropriate for EPA to exempt the TG tree from regulation as a pesticide. It contains no toxicants or pesticidal claims and does not warrant safety instructions or ongoing EPA oversight. Furthermore, due to the transformation vector used, USDA's Animal and Plant Health Inspection Service (APHIS) will fully evaluate its environmental safety before permitting it to be released into commerce or the environment, and a dossier will be submitted to the FDA for food/feed safety evaluation (nuts). These factors satisfy the statutory criteria for EPA to waive the need for pesticide regulation.[2]

Conclusion.

The TG American chestnut is not a pesticide because there is no express or implied intent to mitigate any animate pest, only to minimize physical damage by neutralizing acid. The technology is not directed at the fungus and is indifferent to the continuing presence or absence of the fungus on the tree. The TG tree is intended to coexist with the fungus and the fungus will continue to grow and reproduce on the TG tree.

[2] EPA may exempt by regulation any pesticide adequately regulated by another Federal agency, or of a character not warranting regulation under FIFRA. 7 U.S.C. § 136w(b).

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